Treatment with mesenchymal stem cells in an animal model of parkinson´s disease

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Introduction

Biological aging is characterized by a progressive loss of organism's functions from their physiological optimal values along the time as result of intrinsic and extrinsic factors **(1; 2; 3)**. Several hypotheses may explain the causes of aging but only few are thoroughly accepted. One of them is related to the the loss of capacity of cell replacement in damaged tissues **(4;5).**

Most of the tissues have a specific quantity of adult Stem Cells (SC) responsible for their periodic renovation and regeneration **(6; 7)**. SC have the ability to self-renew by symmetric divisions and the potentiality to differentiate into other cell types depending on their multipotency (**9; 10**). The understanding of SC viability and regenerative capacity is one of the pillars of Regenerative Medicine **(8)**. One type of adult SC are the mesenchymal SC derived from adipose tissue (ADSC), which have been used in our study. There are several procedures by which ADSC can be used to repair and regenerate tissues. For instance, ADSC transplantation into damaged tissues provokes a cytokine- and growth factor-induced response which stimulate their recovery ought to a SC microenviroment (niches) modulation caused by the ADSC presence. This could stimulate the differentiation of endogenous stem cells according to their lineage pathway.

In this paper, the therapeutic potential of ADSC in Parkinson Disease (PD) has been studied in animal models by an intracranial injection of bacterial Lipopolysaccharide (LPS) in the substantia nigra (SN). This brain area, which is rich in dopaminergic neurons, is degenerated in PD. LPS-mediated dopaminergic neurotoxicity depends on the microglial activation, which releases pro-inflammatory factors (NO, TNF-α, IL-1β) **(11; 12)**. Moreover, it was found that the neurotoxicity was mainly mediated by the activation of microglial NADPH-oxidase, generating ROS and neurotoxic factors **(12)**. The aim of this study is to show how stem cells engraftments in an animal model of PD can produce a histological and functional improvement.

Material and methods

Adipose stromal vascular fraction isolation

The stromal vascular fraction (SVF) was obtained from adipose tissue removed from lumbar or abdominal area of Wistar rat anesthetized with chloral hydrate 6%. Adipose tissue was washed with phosphate buffer saline (PBS) to eliminate as much blood as possible.

The tissue was enzymatically processed by a

collagenase solution incubated at 37° C for 150 minutes on an orbital shaker. Then, an equal volume of "stop medium" (Dulbecco's Modified Eagle Medium-DMEM) with 10% fetal bovine serum (FBS) was added and samples were centrifuged at 600xg for 10 min at 4ºC. The pellet (SVF) was resuspended in DMEM + FBS + antibiotic and the cell suspension was filtered through a 40 μm pore-size filter.

Magnetic separation of the ADSC using the CD90.1 MicroBeads.

In order to perform the extraction of the ADSC from a cellular suspension of SVF, a magnetic separation system based on MACS© technology (Magnetic Cell Sorting) were used. This procedure is based on the use of the so-called "microbeads" which are a magnetic micro-particles associated with antibodies that specifically recognize antigens on surface of stem cells. In our case, the CD90.1 (THYmocyte differentiation antigen-1.1) Microbeads antibody was chosen. After magnetic separation, cells were centrifuged, resuspended and cultivated with DMEM+FBS+antibiotic and finally incubated at 37 ° C.

The Countess® Automated Cell Counter was used to determine the number of cells using the Trypan Blue assay. To verify if obtained cells are ADSC, the presence of CD90.1 and CD29 surface markers and the absence of CD11b, CD34, CD45 was checked by flow cytometry, using monoclonal antibodies labeled with fluorophores (mouse antiCD34-PE, CD45-PE, CD11b-PE, CD29-APC and CD90.1-FITC).

***In vivo* tracking of ADSC.**

In vivo tracking systems using gene reporters is a useful tool for cell tracking before, during and after cells engraftment. Optical imaging technologies combined with the use of genetically encoded fluorescent and luminescent proteins have enabled the visualization of stem cells over *in vivo* and *ex vivo* periods of time.

ADSCs were infected with pSIN-DUA-Luciferasa-GFP2 lentivirus with 8 μg/ml polybrene for 24 h. The percentage of GFP-positive cells was determined by flow cytometry. Bioluminiscence activity was measured *in vitro* and *in vivo* using Xenogen IVIS Lumina II imaging system incubation with D-Luciferin (150 μg/ml). For the *in vivo* study, D-Luciferin was injected intraperitoneally at a dose of 150 mg/kg body weight.

Surgical procedure and injecion of LPS and stem cells.

Rats were anesthetized with chloral hydrate (400 mg/kg) and positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA) in accordance with the brain atlas of Paxinos and Watson (1986). Intracerebral surgery was performed by trepanation of the skull and injections into SN were made 5.4 mm posterior, 1.8 mm lateral, and 8.3 mm ventral to bregma (Fig. 1.). Animals received 2 μl of vehicle (1% Monastral Blue) inert tracer in saline or 2 μl of LPS (8 μg) (n = 4) or 2 μl of ADSC solution + LPS (3 x 10^5 cells + 8 μg) in the SN. Experiments were carried out in accordance with the Guide-lines of the European Union Council following the Spanish regulations for the use of laboratory animals and approved by the Scientific Committee of the University of Seville.

Figure 1. Intracerebral surgery

Functional study of learning in operant conditioning chamber (Skinner box).

The experiment conducted in a Skinner box was as follows: the animal was starved for 24 hours. Later, once the rat pressed the lever, a mechanical device drops a pellet of food to the feeder installed inside the same box. After a period of time, the rat activates the lever and the food falls into the feeder. Rat eats pellets and returns to the lever. The process is repeated with insistence and rat runs incessantly from the feeder to the lever. The scheme of learning is the following:

S (conditioned Stimulus)→ R (conditioned Response)→ C (Consequence or Enhancer)

Goal-oriented learning

S→R→C

Habit learning

S→R

S: lever inside the box. R: pressure on the lever.

C: pellet of food

The rats were trained with programs of fixed and variable series which result in a learning goal through enhancers. As a consequence of overtraining, the rats learnt a habit (i.e., some it is done unconsciously). On other hand, we could check if the animal kept pressing the lever even when the enhancer was devalued.

Immunohistochemical analysis

After perfusing the animal with intracardiac injection of paraformaldehyde, frontal lobes were removed. Thaw-mounted 20 μm coronal sections were cut with a cryostat at 20 °C and mounted on gelatin-coated slides. The primary antibodies used were rabbit-derived anti-tyrosine hydroxylase (anti- TH, Sigma; 1:300) and mouse-derived OX-6 (Serotec, Oxford, U.K.; 1:200). OX-6 is directed against a monomorphic determinant of the rat major histocompatibility complex (MHC) class II antigens, expressed by activated microglia but not by the resting cells. Incubations and washes for all the antibodies were in Tris-buffered saline (TBS), pH 7.4. All work was performed at room temperature. Sections were washed and then treated with 0.3% hydrogen peroxide in methanol for 20 min, washed again, and incubated for 60 min in a humid chamber in a solution containing TBS and 1% horse serum (Vector Laboratories, Burlingame, CA, USA) for OX-6 immunostaining or goat serum (Vector) for TH immunostaining. Slides were drained and further incubated with the primary antibody in TBS containing 1% horse/goat serum and 0.25% Triton-X-100 for 24 h. Sections were then incubated for 2 h with biotinylated horse anti-mouse IgG (Vector, 1:200) for OX-6 immunostaining or biotinylated goat anti-rabbit IgG (Vector, 1:200) for TH immunostaining. The secondary antibodies was diluted in TBS containing 0.25% Triton-X-100, and prior to its addition three 10-min rinses in TBS were performed. Sections were then incubated with ExtrAvidin1-Peroxidase solution (Sigma, St Louis, MO, USA; 1:100). The peroxidase was visualized with a standard diaminobenzidine/hydrogen peroxide reaction for 5 min.

For the measurement of the areas expressing OX-6 and TH, the AnalySIS1 image software (Soft Imaging System GmbH, Mu¨ nster, Germany) coupled to a Polaroid DMC camera (Polaroid, Cambridge, MA, USA) attached to a Leika light microscope (Leika Mikroskopie, Wetzlar, Germany) was used. For each animal, five sections (sampling fraction 1:3) were

systematically sampled along the anterior-posterior axis from a random starting point, following stereological criteria. The number of TH-positive neurons in the SN was estimated using a fractionator sampling design. Counts were made at regular predetermined intervals (x = 150 μm and y = 200 μm) within each section. An unbiased counting frame of known area (40 μm x 25 μm = 1000 $\mu m^{2)}$ was superimposed on the tissue section image under a 100x oil immersion objective. Therefore, the area sampling fraction was 1000/(150 x 200) = 0.033. The entire z-dimension of each section was sampled; hence, the section thickness sampling fraction was 1. In all animals, 20 μm sections, each 100 μm apart, were analyzed; thus, the fraction of sections sampled was 20/100 = 0.20. The number of neurons in the SN was estimated by multiplying the number of neurons counted by the reciprocals of the area sampling fraction and the fraction of section sampled.

Statistical analysis

Results are expressed as mean ± SD. Means were compared by One-Way ANOVA followed by the LSD test for post hoc multiple range comparisons. An alpha level of 0.05 was used. The IBM SSPS Statistics 21 package was used for the analyses.

Results

Transfection of ADSC with lentivirus

Transfected cells reached the confluence in 2 days. Then, the percentage of infected cells was determined by flow cytometry.

On the density plot diagram (Fig. 2A), each dot or point represents an individual cell that has passed through the instrument. Cells have been gated as region 1 (R1). 'Region' simply refers to an area drawn on a plot displaying flow cytometry data. On the histogram plot (Fig. 2B) cells are now plotted as Counts on the y-axis versus GFP fluorescence on the x-axis. The percentage of GFP^+ cells were 69,2%.

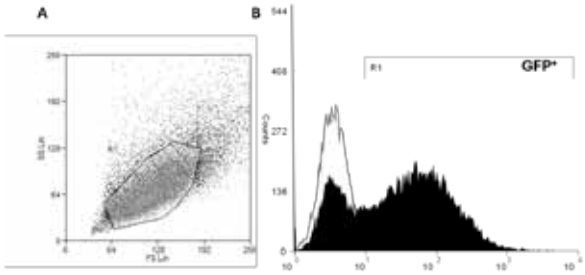


Figure 2. Density plot diagram (A) and Histogram plot of GFP expression (B). Measurement by flow cytometry in channel FL1.

"*In vivo*" tracking of the ADSC.

Bioluminiscence activity in vitro and vivo was determinated using Xenogen IVIS Lumina II imaging system and 3 x 10^5 cells in SN (5.5 mm posterior, 1.5 mm lateral, and 8.3 mm ventral). The injected cells emited 1.72 x 108 ± 5.83 x 107 p/s / cm2/sr at 5 min of exposure time (Fig. 3). This result shows the possibility of *in vivo* tracking in central nervous system.

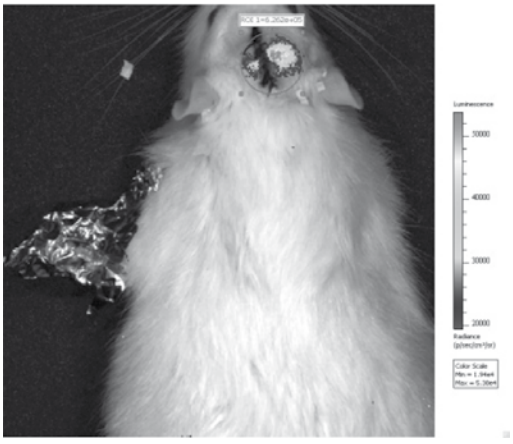


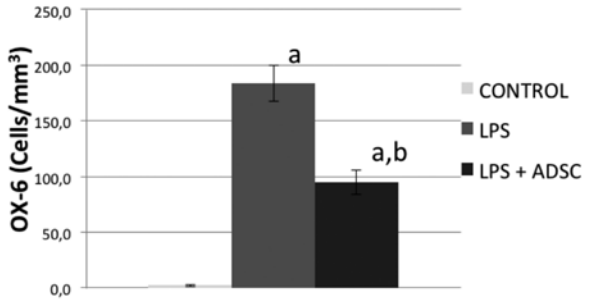
Figure 3. Photographs of optical bioluminescence 1 day after transplantation (1,721·108 p/s/cm²/sr).

Effect of LPS on the microglial population.

In response to certain brain damage, either immune or inflammatory stimulus, microglia suffers significant morphological and metabolic changes. The activated microglia increases the expression of certain proteins, forming the so-called activated microglia **(13, 14)**. Thus, this study aims to study the degree of activation of microglia in the SN in response to LPS injection by using the OX-6 antibody, which only marks the activated microglia.

Injection of vehicle into the SN induced only a slight OX-6 immunoreactivity around the injection track (Fig. 4). Besides, injecting LPS induced a clear microglial activation. LPS+ADSC injections prevented by 50 % this activation.

Figure 4. Quantification of the area expressing OX-6 immunoreactivity in the SN. Results are mean ± SD. aStatistical signification compared with control and bstatistical signification compared with LPS group p< 0.01.



Effect of LPS on the number of TH positive neurons.

The injection of vehicle did not affect the normal pattern of TH immunostaining in these structures (Fig. 5). The number of TH-positive neurons in the SN diminished significantly after the injection of LPS. However, in this case the decrease was partially limited thanks to the ADSC, because the results obtained have a higher standar deviation (no entiendo muy bien en sentido de esta frase). This result is consistent with previous results **(15).**

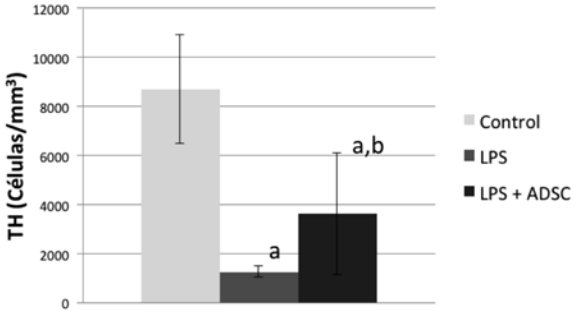


Figure 5. Quantification of the area expressing TH immunoreactivity in the SN. Results are mean ± SD. aStatistical signification compared with control and bstatistical signification compared with LPS group p<0.05.

Functional study of learning.

Operant conditioning (also known as instrumental conditioning) is a process by which organisms learn to behave in such a way as to obtain rewards and avoid punishments. Habit learning is the process by which new behaviors become automatic. If an.acquired behavior is regularly followed, it becomes almost involuntary.

To know if the injuries done in SN affect the dopaminergic activity by the neostriatum (striate dorsomedial and dorsolateral in rodents) we must take into account that the dorsomedial part is related to learning goal behaviours while the dorsolateral part is related to habits **(16).** Using the Skinner box, our results show that control animals acquired the skill in less time than both LPS- and LPS + ADSC-treated animals (Fig. 6).

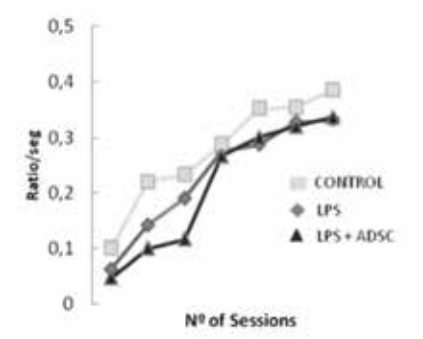


Figure 6.Learning acquisition time.

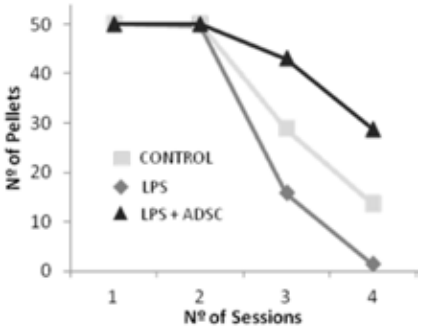


Figure 7. Decline of consumption of the devalued pellet.

To break the R→C connection in the operant conditioning and habit learning we used food pellet of LiCl that produce gastric discomfort to the animal so that they show an aversive behavior to eat it. Using these pellets we can assess whether the learning behavior was predominantly aimed at a goal or maintained by habit. As we can be seen in Fig. 7, LPS-treated animals quickly lower their rate of response, which indicate that their learning is primarily aimed at a goal. It is worthy to note that LPS + ADSC animals showed a higher rate of response than control subjects, which suggests a greater ability to acquire a habit.

Discussion

Cell therapy has been studied in a model of Parkinson's disease (PD). The working hypothesis is that the ADSC, implanted locally, can recognize the damage and stay in these tissues during a period of time and, later, contribute to their reparation. The idea is that autologous treatments with ADSC would prevent or treat the loss of optimal neurophysiological functions occurring with age.

An LPS experimental model of PD was used. LPS induces a strong reaction in the microglia as indicated by a 67-fold increase in the OX-6 compared to control data. The SN has special sensitivity to the inflammation stimulus due to the fact that it contains the highest density of microglial cells **(13)**. In addition, LPS led to a loss of more than **70%** of TH positive neurons. It has been described that the increase in the synthesis of inflammatory cytokines by glia produces the selective death of dopaminergic neurons due to oxidative stress **(18, 19, 20)**. Considering the inflammatory process that occurs in the SN after injection of LPS **(15)**, it has been suggested that inflammation could be involved in certain neurodegenerative processes such as PD.

The results also show that ADSC partially prevented both the microglial activation caused by LPS and the loss in the number of TH-positive neurons. Likewise, ADSC treatment led a slight tendency to observe a greater ability to acquire a habit. In conclusion, stem cells are capable of improving inflammation, neuronal death and possible improvements at the functional level in PD.

Acknowledgement

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